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A Convenient Method for the Synthesis of Peptides Acylated with Palmitic Acid Specifically at Cysteine Thiol

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Abstract

Acylation of a cysteine containing peptide with palmitoyl chloride, when attached to a solid support, followed by acidolytic cleavage of the peptide from the resin, to generate the fatty acylated peptide was explored. Our results indicate that this is a convenient method to synthesize peptides specifically acylated at the thiol group of cysteine.

In recent years, several proteins have been found to be acylated with fatty acids[1-4]. The amino acids to which fatty acids are covalently attached are glycine at the alpha-amino group via an amide bond[5] and cysteine at the -SH group via a thioester bond[6-8]. In vivo, acylation is accomplished by enzymes[9,10]. Many fatty acylated proteins are either oncogene products[11] or growth factor receptors[12] and it would be of considerable interest to know the role of this post-translational modification. Peptides farnesylated at thiol groups of cysteine have helped in identifying and characterizing farnesyl-protein transferases[13-15]. Also, synthetic peptides, myristoylated at the N-terminus have aided in identifying possible receptors in cells[16]. In order to elucidate the manner in which a covalently linked palmitic acid would modulate the association of a hydrophobic peptide with membranes, as well as identify receptors for palmitoylated segments of proteins, it would be desirable to generate peptides with regioselective palmitoylation at cysteine thiols. Selective acylation at the thiol group of C is not easy in fully deprotected peptides, especially when amino acids like K, S and T are present. We have explored acylation of peptides at -SH of C when attached to solid supports, followed by acidolytic cleavage of the acylated peptides from the resin, to generate fatty acylated peptides.

Synthesis of WKTPGCVKIKKA corresponding to one of the ras related GTP binding protein[17] was accomplished on a Pharmacia semi-automated synthesizer using fluorenylmethoxycarbonyl (Fmoc) chemistry, ultra Syn KA resin from Pharmacia and

continuous flow conditions. Solvents were from Merck India. Trifluoroacetic acid (TFA), thioanisole and metacresol were from Fluka. Ethanedithiol was from Sigma, USA. All protected amino acids were from Nova Biochem, U.K. The side chain protecting groups were K (t-butyloxycarbonyl), T (Butyl) and C (acetamidomethyl, Acm). The first amino acid was attached by the symmetric anhydride method[18]. 1 gm of the resin with a substitution of 0.09 mmol of alanine was used for the synthesis. One cycle of operation consisted of the following steps:

(i) deprotection, 20% piperidine in dimethylformamide (DMF), 10' (3 ml/min), (ii) wash, DMF, 10' (3 ml/min), (iii) addition of preformed 1-hydroxybenzotriazole (HOBT) active ester (prepared by reaction of 1 eq. of HOBT and dicylohexylcarbodiimide (DCC) with Fmoc amino acid in DMF), 4 equivalents and recycling for 1 hr, (iv) wash with DMF, 10' (3 ml/min). After the synthesis, the amino terminal group was acylated with acetic anhydride[18]. The Acm group was removed with mercuric acetate as follows: The acylated peptide bound to the resin (100 mg) was suspended in 2 ml of 30% acetic acid and two equivalents of mercuric acetate to every equivalent of Acm was added. The reaction mixture was shaken gently for 1 hr at room temperature. Twenty equivalents of 2-mercaptoethanol was added to the reaction mixture to precipitate the excess mercury as mercuric sulphide. Shaking was continued for 1 more hr. After decanting the acetic acid and suspended mercuric sulphide, the resin was washed 2-3 times sequentially with t-amylalcohol, ether and DMF. The resin was then suspended in DMF and two equivalents of palmitoyl chloride was added followed by 2 equivalents of diisopropylethylamine. The reaction was allowed to proceed for 12 hrs. At the end of this period, the resin was washed with chloroform and ether. The peptide bound resin was dried and the fatty acylated peptide was cleaved from the resin with TFA : thioanisole : metacresol : ethanedithiol (10:1:1:1). Evaporation of trifluoroacetic acid followed by trituration with ether yielded the peptide as a white powder (8 mgs of peptide was obtained which corresponds to a yield of $\sim$70%). The acylated and non-acylated peptides were purified by Fast Performance Liquid Chromatography (FPLC) on a reverse phase pep RPC 5/5 (Pharmacia) column. The FPLC profiles of purified non-fatty acylated and fatty acylated peptides are shown in Figs. 1(a) and (b). Acylation with fatty acid results in increased retention time as expected, as fatty acid acylation would render the peptides more hydrophobic. Comparison of the FPLC profiles of crude fatty acylated and non-fatty acylated (but C with acetamidomethyl group) peptides indicated that the efficiency of deprotection of the Acm group was 90%. The pure acylated peptide was hydrolyzed with 6N HCl for 24 hrs. After hydrolysis, the aqueous soluable part was used for amino acid analysis. The insolua-

ble portion was extracted with $CHCl_3$ and after evaporation of $CHCl_3$, the residue was treated with diazomethane and subject to gas chromatographic (GC) analysis. Amino acid analysis on a LKB 4151 Alpha Plus Amino Acid Analyzer yielded T 1.2 (1), P 1.0 (1), G 1.2 (1), A 1.18 (1), V .88 (1), I .83 (1), K 3.83 (4). Numbers in parantheses indicate theoretical values. The amino acid W and C were not quantitated due to destruction during hydrolysis. Presence of W was confirmed by UV and fluorescence spectroscopy. Estimation of peptide concentration by UV correlated well with amino acid analysis. Quantitation of fatty acid on a Hewlett Packard 5840 A gas-liquid chromatograph indicated that the peptide contained molar equivalent of palmitic acid. Over all yields of the purified fatty acylated and non-acylated peptides were ~65% from the crude.

Our results indicate that fatty acid acylation at cysteine after removal of Acm protecting group on peptides bound to resin and subsequent deprotection to remove side chain protecting group of amino acids other than cysteine and purification, is a convenient method to generate peptides acylated specifically at cysteine residues.

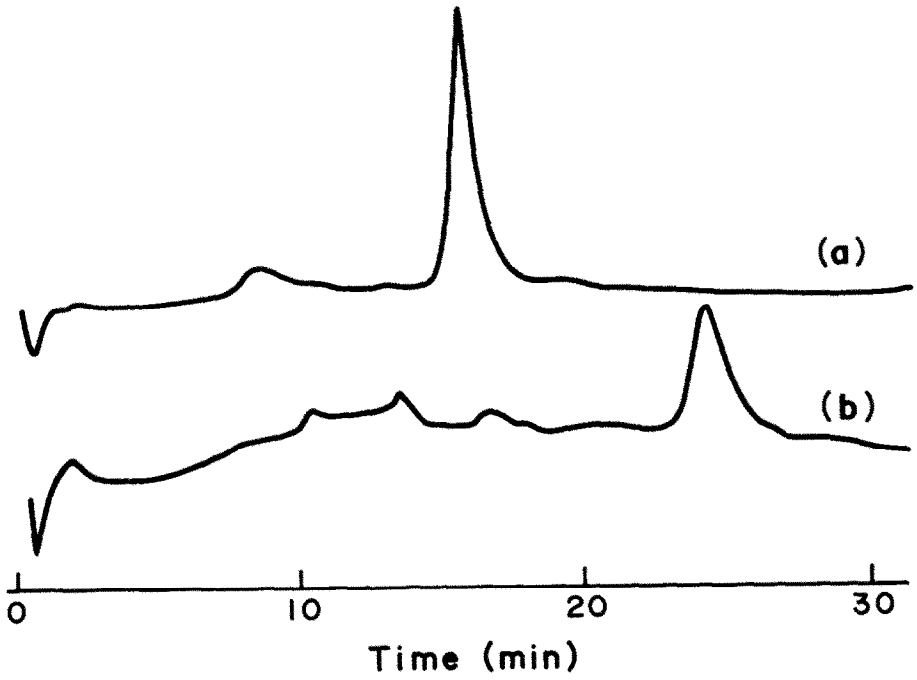

Fig. 1 FPLC of non-acylated and fatty acylated peptides. (a) non-acylated, (b) fatty acylated. Conditions : 0-15% CH_3CN having 0.1% TFA (solvent B) in 8 min (solvent A = 0.1% TFA in H_2O) followed by isocratic (15% B) elution for 22 min. Flow rate was 0.5 ml/min through a Pharmacia PepRC 5/5 reverse phase FPLC column. Detection was at 214 nm.

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